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An epidemiologic approach to studying heterocyclic amines

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Abstract

Diets containing substantial amounts of red meat may increase the risk of colorectal, pancreatic, breast, prostate, and renal cancer. The association with red meat intake may be due to a combination of factors, such as content of fat, protein, and iron, and/or meat preparation (e.g. cooking or preserving methods).

Laboratory results have shown that meats cooked at high temperatures contain heterocyclic amines (HCAs) known to be mutagenic and carcinogenic in animals. Many older epidemiologic studies of colon cancer using surrogates for HCA exposure from meat (for example, doneness level, surface browning, frying, intake of gravy) have produced suggestive but inconsistent results. These discrepancies may have resulted in part from having used dietary questionnaires that combined meat-cooking practices in ways that made the intake of HCAs difficult to estimate. Thus, over the last decade we have taken a multidisciplinary approach to investigating whether the association with red meat intake can be explained by meat-cooking practices that produce mutagens/carcinogens. To estimate intake, a database for HCAs have been developed and used in conjunction with a validated meat-cooking food frequency questionnaire (FFQ). To develop biological markers of internal exposure, a metabolic study was conducted where subjects consumed controlled amounts of meat cooked at low and high temperatures.

The role of meat type, cooking methods, doneness levels, and meat-cooking mutagens were examined in case-control studies of colorectal adenomas, lung, and breast cancers using both questionnaire information and biomarkers. In a case-control study of colorectal adenomas, an increased risk was associated with a high intake of red meat. Most of this risk was due to intake of red meat cooked until well/very well done and/or by high-temperature cooking techniques such as grilling. Linking the FFQ information to HCA database, the impact several HCAs on risk was evaluated. An increased risk was associated with higher intake of MeIQx, possibly PhIP. Red meat, especially fried and/or well-done red meat, was associated with increased risk of lung cancer in a population-based case-control study. In addition, an increase in risk was demonstrated among non-smokers and moderate smokers for MeIQx intake. In a case-control study of breast cancer well-done red meat and PhIP was associated with increased risk of breast cancer. In this manuscript I will provide one approach to studying the relation of meat cooking-mutagens and cancer risk and will suggest the types of studies that may be required in the future to clarify these associations.

Keywords: Heterocyclic amines; Cooking method; MeIQx; PhIP

1. Overview

Experimental studies have demonstrated that meat cooked at high temperatures contained heterocyclic amines (HCAs), which are mutagenic and carcinogenic in animals. Early epidemiologic studies conducted

Abbreviations: HCAs, heterocyclic amines; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

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in the 1980s suggested an association between meat-cooking techniques and cancer risk. However, in these studies information obtained on meat-cooking practices could not differentiate factors that had an important influence on the production of HCAs. For example, roast beef and steak were included within the same meat category, despite widely dissimilar cooking methods and very different levels of HCA formation. Before initiating etiologic studies, tools were needed to estimate intake of HCAs in an accurate and reliable manner.

The two approaches to estimating dietary exposure to HCAs that I used were: (1) questionnaire-based measures of intake; and (2) assessing biological markers of HCAs.

My research focuses on three main HCAs as they were the one that were detected in the meat samples:

- 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx);
- 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx); and
- 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP); (IQ). 2-amino-3-methylimidazo[4,5-*f*]quinoline.

This review of my research is divided into three parts. The opening section explains the multidisciplinary approach (Fig. 1) used to investigate whether the cancer association with red meat intake can be explained by meat-cooking practices that produce mutagens/carcinogens, including current status and future needs in exposure assessment of HCAs. The middle section provides results from epidemiologic studies using these newer methods of estimating HCA intake. The closing section explores directions for future research.

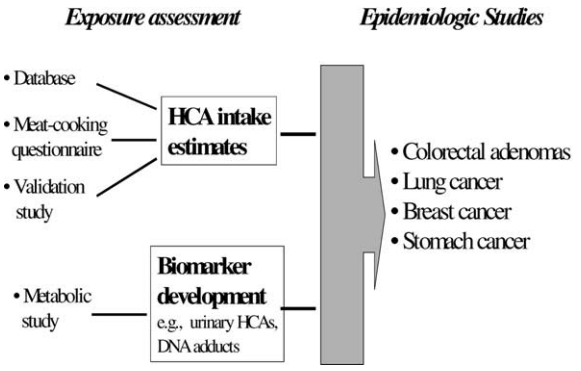

Fig. 1. Multidisciplinary components of the HCA research program.

2. Heterocyclic amines exposure assessment

To estimate intake, we developed a database for HCAs, which could be used in conjunction with a validated meat-cooking food frequency questionnaire (FFQ). To develop biological markers of internal exposure, I carried out an intensive metabolic study where subjects consumed controlled amounts of meat cooked at low and high temperatures.

2.1. Development of methods to estimate HCA intake

To date, no attempt had been made to conduct an extensive and systematic evaluation of meat types and cooking methods with the purpose of measuring HCA production. We undertook this by developing a database for HCAs in conjunction with a meat-cooking questionnaire.

To estimate HCA intake we first needed to develop a HCA database and questionnaire and then needed to validate it (Fig. 2).

2.1.1. HCA database

The HCA database was developed in conjunction with a FFQ meat-cooking module. For each meat line-item in the questionnaire, multiple samples were cooked by different methods to varying degrees of doneness. Approximately 2500 individual pieces of meat were cooked to provide data in 120 categories by cooking method and doneness. These data were ultimately used to create the HCA database.

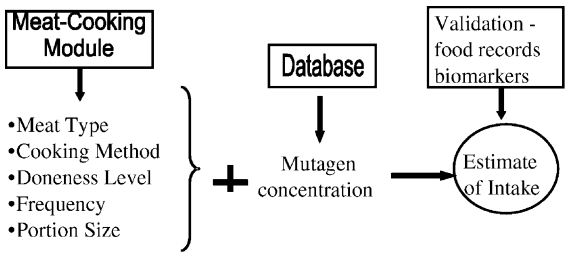

Fig. 2. Method used to estimated intake of HCAs.

We assessed mutagenic activity with the Ames *Salmonella* test and HCA levels as measured by high pressure liquid chromatography (HPLC). The details of HCA content in the various types of meat samples are contained in several published reports [1–5].

In developing the database [1–5], we found that:

(1) IQ and MeIQ were not detectable in any of the meat samples;
(2) measured values of the specific HCAs varied with meat type, cut of meat, cooking method, and doneness level;
(3) different HCAs were formed in varying amounts, with PhIP being the most abundant and DiMeIQx the least abundant;
(4) HCAs generally increased with doneness; and
(5) very high levels of PhIP are formed in chicken cooked to very well done by specific methods.

These results suggest that questions on meat intake not including details of type or cut of meat, cooking technique, and doneness level are likely to misclassify HCA exposure. For the same level of doneness, steaks, hamburger patties, and roast beef had substantially different levels of HCAs.

Moreover, from our published data it appears that even within the same type of meat and doneness level, place of cooking (for example, fast-food restaurants, non-fast-food restaurant, and home), or cooking method (pan-fry, oven-broil, and grill/barbecue) played a major role in determining HCA content [6]. The three high-temperature cooking methods (pan-frying, oven-broiling, and grilling/barbecuing) produced varying levels of HCAs. Pan-frying and grilling/barbecuing produced more MeIQx, DiMeIQx, and PhIP, while oven-broiling produced very little of these compounds.

2.1.2. Meat-cooking FFQ module

Given the above findings, we then began to develop a meat-cooking module within a FFQ format that could better estimate HCA intake. By reviewing the published literature and developing the HCA database, it became clear that HCA production resulted from two important elements of meat cooking: temperature and time. We also found that cooking technique could serve as a reasonable proxy of temperature and doneness level as a surrogate for time. Using existing FFQs, I embedded the cooking methods and doneness levels within each meat line-item. When necessary, certain categories were separated to reflect meat types cooked by different techniques. For example, in previous FFQs, roast beef and steak had been included within the same meat category, despite widely dissimilar cooking methods and levels of HCA formation.

2.1.3. Validation of the meat-cooking FFQ module

To validate the meat-cooking module, I designed a study among the 156 healthy controls participating in a breast and prostate cancer study. The volunteers completed a 24 h recall and a FFQ including detailed questions on meat-cooking practices. Subjects kept food-diaries for 12 days over a 3-month period and recorded how they cooked meat, including its appearance inside and outside. The concordance for ever/never consumption for different types of meat was between 70 and 90%. The correlation between certain macro- and micro-nutrients estimated from the FFQ and mean values from the food-diaries was similar to that observed in other validation studies, ranging from 0.4 to 0.6. However, the correlation between FFQ and food-diaries for PhIP were lower. These results suggest the need to improve the way questions are asked about certain types of meat, especially chicken, because chicken cooked in certain ways can contain large amounts of PhIP.

2.2. Development of biomarkers of HCA exposure in a metabolic study

Thirty-three men and 33 women were selected to participate in a metabolic study with two 7-day controlled dietary periods [7]. The first period contained meals with meat cooked at low temperatures while the second period contained meat cooked at high temperatures. Meat cooked at low temperature did not contain detectable levels of HCAs while meat cooked using high-temperature methods contained high amounts of HCAs. The amount of meat consumed by each subject varied with his or her individual body weight. Blood, urine, and fecal samples were collected at multiple times over the experimental period. The subjects underwent phenotyping for CYP1A2 and NAT2 activity by measuring the ratio of urinary caffeine metabolites at three times: when they entered the study, at the end of the low-temperature cooked meat period, and at the end of the high-temperature cooked meat period.

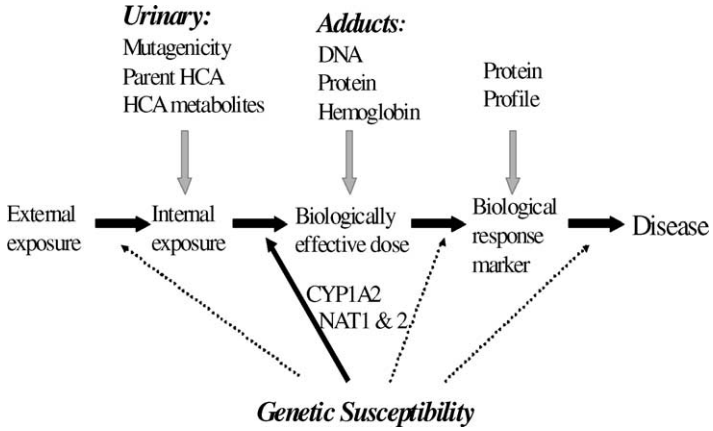

Fig. 3. HCA biomarkers and polymorphic enzymes involved in HCA metabolism.

Fig. 3 outlines the potential types of HCA biomarkers (e.g. marker of exposure, marker of biologically effective dose and response) investigated.

2.2.1. HCA urinary biomarkers as a measure of internal exposure

Several markers of HCA intake in the urine were measured using samples from the end of the low-temperature cooked meat period and throughout the high-temperature cooked meat period. The different markers were: mutagenic activity measured by Ames *Salmonella* test; HCAs (free and acid hydrolyzed); and metabolites of HCA [8–11].

Urinary free HCAs or total (acid-hydrolyzed urine samples includes free, N^2-glucuronide, and sulfamate metabolites) HCAs correlated modestly with the amount of HCA consumed in the metabolic study, with the Spearman correlation coefficients between 0.4 and 0.6. HCAs and mutagenic activity in the urine were not detectable 12 h after consuming the high HCA meal. This indicates that HCAs in urine have short half-lives and may not be ideal measures of "usual" intake in etiologic studies, especially if there is substantial day-to-day variability. However, with a large sample size, it could still be used to validate intake of HCAs as estimated by questionnaires.

2.2.2. HCA-adducts as a measure of biologically effective dose

DNA-adducts measured in various laboratories using ^{32}P-postlabeling methods have not been sensitive enough to detect a signal, even in subjects who consumed high levels of MeIQx and PhIP during the high-temperature cooked meat period. Thus, at present, it is unlikely current methods will be able to measure HCA-adducts in a free-living population, whose meat intake is likely to be much lower than the meat intake of subjects in the metabolic study. However, with newer technologies long-term biomarkers such as DNA-, protein-, and hemoglobin-adducts will be developed in the future.

2.2.3. Polymorphic enzymes involved in HCA metabolism

The cancer risk posed to humans by dietary HCAs may depend on the extent to which the compounds are activated in vivo. The initial activation step is thought to be *N*-oxidation by liver CYP1A2 [12]. The *N*-hydroxylamine metabolite either undergoes *O*-acetylation in the liver or the metabolite is transported to the appropriate target organ, where it undergoes acetylation by the polymorphic acetyltransferases, NAT1 or NAT2. The acetylated compound can form adducts which lead to DNA damage. CYP1A2 and NAT2 activities can be measured by evaluating excretion of caffeine metabolites in urine after caffeine consumption [13]. The measured phenotype distinguishes between slow and rapid *N*-oxidizers and *O*-acetylators.

In the metabolic study, CYP1A2 and NAT2 activities were measured at baseline and at the end of the low- and high-temperature cooked meat periods. NAT2 activity remained unchanged throughout the

study, while CYP1A2 activity increased in most of the subjects after consuming high-temperature cooked meat, suggesting induction by some compound(s) formed during high-temperature cooking. There was a high within-person correlation for CYP1A2; subjects with low activity after eating low-temperature cooked meat tended to stay relatively low even after consuming high-temperature cooked meat and the subjects who were high tended to stay relatively high. This suggests a fixed component in the regulation of CYP1A2 and the need to consider both a fixed and an inducible component of this enzyme in epidemiological studies. The different genetic polymorphisms identified in the *CYP1A2* gene have not been associated with the induction observed during the high-temperature cooked meat period.

We then examined the relationship between CYP1A2 and NAT2 activity, and excretion of free HCA and metabolites in subjects from the metabolic study. We found that higher CYP1A2 activity was associated with lower levels of free MeIQx in the urine when adjusted for amount of meat eaten, while NAT2 activity showed no relationship with the latter [8]. This suggests that a greater percentage of MeIQx may be converted to metabolites such as the *N*-hydroxy derivative when CYP1A2 activity is higher. However, there was no relationship between urinary *N*-OH-MeIQx–N^2-glucuronide and CYP1A2 or NAT2 [11].

2.2.4. Future directions for improving HCA intake estimates

Questionnaire: Intake estimates based on questionnaire data may be the best method of assessing HCA exposure in the near future. While, there is room for improvement in both the food frequency questionnaire and HCA database, these changes are unlikely to dramatically improve exposure estimate. It may be useful to collect duplicate food-plates, complete 24 h recalls, or use diaries to estimate HCA intake, but the cost for these studies are extremely high.

Biomarkers: HCAs excreted in the urine either as parent compounds or metabolized molecules are short-term markers, reflecting intake within the last 24 h. Short-term markers are useful tools for studying mechanism of action of HCAs and for validating questionnaires. However, in order for biomarkers to be a viable exposure assessment tool for epidemiologic studies long-term biomarkers, such as DNA-, protein-, hemoglobin-adducts which reflect intake over weeks or months are needed.

3. Cancer epidemiologic studies of meat intake, cooking techniques, and HCAs

Evidence from epidemiologic studies examining the relationship between meat intake and cancer and from HCA animal carcinogenicity studies indicates that several cancers may be related to HCA intake. The evidence is strongest for colorectal cancer, however, other data also suggest some role for HCAs in other tumors including breast, pancreatic, prostate, lung, hepatic, and renal cancers as well as lymphomas.

3.1. Meat and HCA intake estimates from studies in US populations

HCA consumption per day is estimated to be on the order of nanograms based on data from several studies in the US [14–20]. Most people in these studies consumed relatively low quantities of HCAs and only subjects in the top quintiles have substantially different intake levels from those in the lowest quintile (Fig. 4) [16]. In contrast, meat consumption varied greatly by region in the US, with median intake of red meat as high as 70 g per day in a lung cancer study of women in Missouri [14] and 7 g per day in a breast cancer study of women from in California [20].

3.2. Colorectal adenoma

Using the meat-cooking module and database described above, we investigated the role of meat intake, meat-cooking techniques, and meat-cooking mutagens in a hospital-based case–control study of colorectal adenomas [16,18]. We found a non-significant increased risk of colorectal adenomas of 4% per 10 g per day (or 2.5 oz per week) of total meat intake. This increased risk for total meat partitioned into a significant 11% per 10 g per day risk increase for consumption of red meat and a non-significant decrease in risk of 5% per 10 g per day for white meat intake [16,21]. These results correlated with the increase in risk with red meat consumption previously reported [18]. We further partitioned risk associated with well done/very

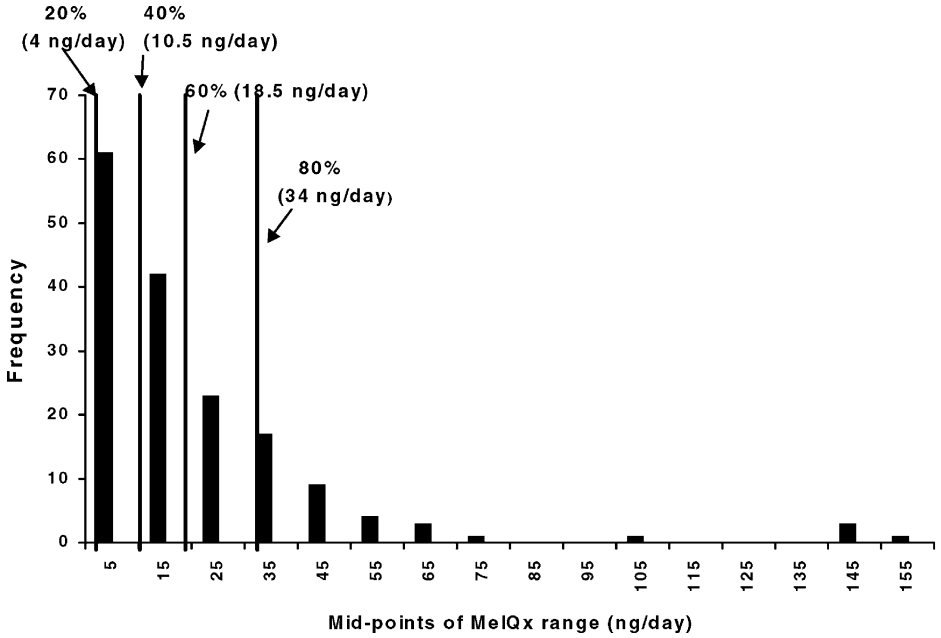

Fig. 4. Distribution of MeIQx estimated from FFQ.

well done red meat and found a 29% risk increase per 10 g per day. Further, high-temperature cooking methods were also associated with an increased risk of 26% per 10 g per day of grilled red meat and 15% per 10 g per day of pan-fried red meat consumption. The risk was further elevated to 85% per 10 g per day among subjects who ate well done/very well done grilled meat.

Risk of colorectal adenomas doubled in the highest compared to lowest quintile of intake of these compounds [16]. The excess risk was confined to the fifth quintile for DiMeIQx and MeIQx, and to both the fourth and fifth quintiles for PhIP. We also investigated the relationship between polymorphisms in genes coding for enzymes involved in the metabolism of HCAs and colorectal adenomas. A small suggestive increase in risk associated with *NAT1* genotype, which is linked with higher activity, was also noted. NAT2 enzyme activities or the fast acetylator *NAT2* genotype showed no association with risk.

3.3. Summary

The research outlined about yielded several important findings. We found an association between red meat intake and increased risk of colorectal adenomas (Section 3.2) [16,18], and lung [14,15] and stomach cancers [22]. Intake of meat cooked by grilling, a possible surrogate for HCAs, increased the risk of colorectal adenomas and stomach cancer, whereas intake of fried meat, which contains mainly HCAs, was associated with lung cancer. With greater degrees of red meat doneness, a proxy for higher level of meat-cooking carcinogens, increase in risks for colorectal adenomas, lung, breast [17,19], and stomach cancers [22] were observed. These data suggest some degree of organ specificity for HCAs and are consistent with those obtained in animal carcinogenicity studies (Table 1) [23].

Using the Bradford–Hill criteria for causal association I have summarized the progress made between 1995 and 2001 in trying to establish the role of well

Table 1
HCA carcinogenicity in animal and epidemiologic studies

Site	Animal studies	Epidemiologic studies
Colon	IQ, MeIQ, PhIP	DiMeIQx, MeIQx, PhIP?
Lung	IQ, MeIQx	MeIQx
Mammary/breast	PhIP	PhIP

Table 2
Criteria for causal association for well done meat and HCA: epidemiologic evidence in 1995 and 2001

	1995	2001
Strength	?	OR around 2
Consistency	Low	Low/medium
Dose–response	?	Some
Biological plausibility	Ample	Ample

done meat and HCA with human cancer (Table 2). Since 1995, the strength of the association has been better demonstrated and is likely to be around 2 with higher levels in subgroups with certain genotypes. Presently, consistency between different studies is low to medium. However, many ongoing studies will add substantially to our knowledge once they are completed. The dose–response relationship is difficult to gauge since most of the population is not exposed to these compounds. Much of the range for HCAs is in the top quintile so measuring dose–response using conventional categorical analyses may not be readily apparent. Studying populations with higher intakes of these compounds may be important to clarify a dose–response relationship. Biological plausibility, the last criterion, is supported by ample evidence from both laboratory and animal studies.

4. Future research

The evidence for a role association of HCA in human cancer is building. However, future research will need to be improved in several ways in order to elucidate the connection. Areas of research needing exploration include: (1) the continuing development of "best" exposure assessment possible using both biomarkers and questionnaires; (2) conducting large studies with case numbers over 2000, especially when investigating the role of genetic polymorphisms; (3) using prospective cohort design to decrease different bias inherent in case–control design, which may be of special concern for nutritional studies; (4) conducting studies in different populations with diverse dietary patterns. These studies could include groups with:

- a wide range of consumption of different types of meat and cooking methods, such as in different European countries;
- rapidly changing diets with increasing meat consumption, such as in Japan and China;
- high meat intake using high-temperature cooking methods, such as in some South American countries and Australia/New Zealand.

One caveat in this research area is to keep in perspective a positive finding that is a result of multiple comparison analysis or "fishing expeditions" as compared to findings generated based on a priori hypotheses and research. Diligent efforts in these research directions will continue to expand our knowledge of HCAs and their role in carcinogenesis.

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